

JOURNAL OF ANIMAL SCIENCE

The Premier Journal and Leading Source of New Knowledge and Perspective in Animal Science

Flow cytometric analysis of porcine preadipocyte replication

J. T. Wright and G. J. Hausman

J Anim Sci 1994. 72:1712-1718.

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://jas.fass.org>



American Society of Animal Science

www.asas.org

Flow Cytometric Analysis of Porcine Preadipocyte Replication

J. T. Wright¹ and G. J. Hausman

Animal Physiology Unit, R. B. Russell Research Center, ARS, USDA, Athens, GA 30613

ABSTRACT: In the present study, a technique is described for measuring proliferation of primary porcine preadipocytes. Cultures of stromal-vascular (S-V) cells from dorsal subcutaneous adipose tissue were maintained under several growth conditions. Cells were isolated from cultures at various times and DNA was labeled with propidium iodide (PI) and analyzed by flow cytometry. Treatment of cultures with colchicine allowed identification of S-V cells in mitosis. The relative proportion of replicating cells

was dependent on several factors, including serum concentration and cell density at the time of harvest. Using the AD-1 anti-preadipocyte/adipocyte monoclonal antibody, the preadipocyte subpopulation within the S-V cells was identified. Furthermore, by labeling cells with PI concomitant with AD-1, the proportion of replicating preadipocytes was quantified. It is now possible to identify and quantify replicating preadipocytes rapidly under various experimental conditions.

Key Words: Adipocytes, Cell Cycle, Pigs, Propidium Iodide

J. Anim. Sci. 1994. 72:1712-1718

Introduction

Measurement of preadipocyte proliferation in tissues as well as in stromal-vascular (S-V) cultures is confounded by the presence of nonadipose lineage cells. In primary S-V cultures, increased cell number indicates replication of at least a proportion of cells, but the replicating proportion may be all, some, or none of the preadipocytes per se (Wright and Hausman, 1990a,1993; Wright 1992). There are very few markers for adipose lineage cells that are effective tools for enumerating preadipocytes during their replicative stage of development. Therefore, replication of primary porcine preadipocytes has not been studied in any detail.

We have previously developed methodology for flow cytometric quantification of preadipocytes from primary S-V cultures by labeling cells with an anti-adipocyte/preadipocyte monoclonal antibody (MAB; Wright, 1992). This MAB, designated AD-1, identifies cells of the adipocyte lineage before onset of lipid deposition in vivo and in vitro (Wright and Hausman, 1990a,b). The present study was designed to characterize the use of propidium iodide (PI), a fluorescent stain for nucleic acids, coupled with flow cytometry to measure replication of primary porcine S-V cells. We also present evidence to demonstrate that PI staining

together with cell surface marker staining using the AD-1 MAB can be used to examine proliferation of preadipocytes.

Materials and Methods

Stromal-vascular cells were obtained from the dorsal subcutaneous adipose depot from 5- to 7-d-old pigs. Pigs were euthanatized by sodium pentothal overdose. Cell suspensions were prepared by collagenase digestion (Bjorntorp et al., 1979) and seeded in 35-mm tissue culture dishes at subconfluent densities of 1.25×10^4 cells/cm² (.5×), 2.5×10^4 cells/cm² (1×), and 5×10^4 cells/cm² (2×) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Basal medium was DME/F12 medium (Sigma Chemical, St. Louis, MO) supplemented with 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 2.5 µg of amphotericin-β/mL. All cultures were initiated in basal medium supplemented with 10% fetal bovine serum (FBS). Adipocyte differentiation media used were basal medium supplemented with 2, 5, or 10% pooled pig serum (PS) and 2% PS supplemented with 10 nM dexamethasone (DEX). Colchicine (.4 µg/mL) was used in some experiments to inhibit mitosis. All animal procedures had prior approval by the USDA-ARS Animal Care Committee.

Flow Cytometry

Preparation of isolated cells from S-V cultures for flow cytometry has been described in detail (Wright,

¹To whom correspondence should be addressed: P. O. Box 5677.
Received December 10, 1993.
Accepted March 11, 1994.

1992). Briefly, cells were liberated from cultures by digestion using .5% collagenase/.5% hyaluronidase in calcium- and magnesium-free (CMF) Hanks' balanced salt solution at 37°C for 10 min followed by addition of EDTA in CMF-Hanks' to a final concentration of 5 mM (Wright, 1992). After 10 additional minutes liberated cells were pelleted by centrifugation, washed using DME/F12 medium, and stained for 20 min with control or the AD-1 MAb (1/250 dilution of ascites), rinsed, and stained with fluorescein isothiocyanate-(FITC) conjugated goat anti-mouse IgG (.1 mg/mL) for an additional 20 min. Stained cells were fixed for 30 min with ice-cold 1% paraformaldehyde in PBS, rinsed, and then permeabilized with .05% Nonidet P-40 in PBS for 3 min. Permeabilized cells were treated with 10 μ g of RNase-A/mL and then the DNA was labeled with 25 μ g of PI/mL immediately before analysis. Flow cytometry was performed using an Epics 753 argon laser flow cytometer (Coulter, Hialeah, FL). In preliminary studies, successful RNase treatment was ascertained by using higher concentrations of RNase-A and by examining stained cultures in situ. The PI was added to fixed and permeabilized cells immediately before analysis in all instances. The effect of fixation on AD-1 immunoreactivity was determined by comparing relative fluorescence of AD-1 on both fresh and fixed cells. The relative proportion of cells stained by AD-1 was unaffected by postfixation, permeabilization, RNase-A treatment, PI staining, and colchicine treatment. However, it was necessary to stain using the AD-1 MAb before, and not after, the fixation step.

The cell cycle is generally considered to be composed of four phases, namely the gap prior to DNA replication (G1), the synthetic phase (S), the gap after DNA (G2), and mitosis (M) (Hartwell and Weinert, 1989). Quiescent, or noncycling, cells would be considered in G0. For quantitative analyses, gating parameters were set to yield G0/G1 (2N), S (intermediate), and G2/M (4N) cell subpopulations based on computer-generated histograms. The position of G2/M cells was verified based on shifts in histograms after treatment of cultures with colchicine. The relative position of cell peaks remained constant among samples within an experiment and absolute mean fluorescence values of peaks were determined by the cytometry computer program for each separate experiment on different days.

Statistics

Cells from three or four dishes were pooled in each experiment for each observation. Flow cytometric profiles shown are representative of typical experiments. Each data set represents four separate experiments with the exception of three separate experiments in the two parameter analysis. All observations for each data set were included in each experiment. Data were subjected to analysis of variance using the

GLM procedures of SAS (1985). Differences were only determined within a given cell cycle phase (i.e., G0/G1 only tested against other G0/G1 values, etc.). Differences between treatment means were determined by least squares contrast (SAS, 1985).

Results

Identification of Replicating Cells

The point at which S-V cells reached confluence in basal medium supplemented with FBS was dependent on seeding density. Representative histograms depicting cell number vs PI fluorescence of cells from cultures initiated at three densities are shown in Figure 1 and results are summarized in Figure 2. At the time of harvest, cultures seeded at .5 \times density (Figure 1A,B) were pre-confluent and those seeded at 2 \times density (Figure 1E,F) were post-confluent, whereas those seeded at 1 \times density (Figure 1C,D) were intermediate. Two subpopulations of cells based on DNA content were primarily apparent at all densities. Treatment of cultures with colchicine (Figure 1B,D,F) increased ($P < .05$) the proportion of cells with peak immunofluorescence (IRFL3) of 138 (hereafter referred to as G2/M cells) with primarily a concomitant diminution in the relative proportion of cells with peak fluorescence of 72 (hereafter referred to as G0/G1) cells (Figure 1). The G2/M cell subpopulation in this series of experiments included cells with IRFL3 values from 120 to 179 (computer-forced baseline) and cells with the mean PI fluorescence value for the peak at 72 in Figure 1 including cells with IRFL3 values from 51 to 96 were G0/G1 cells. The midpoint (107) was median for S phase cells and a range for S was from IRFL3 96 to 118. In FBS cultures, colchicine increased G2/M cell number independent of density (Figure 2).

Replication of Stromal Vascular Cells in Differentiation Medium

Stromal vascular cells were seeded at 1 \times density and grown for either 24 or 72 h in medium containing 10% FBS and an additional 24 h in medium supplemented with 2% PS/DEX, a relatively common and potent stimulator of lipid deposition by preadipocytes (Hentges and Hausman, 1989; Gaskins et al., 1990; Wright, 1992). At the time of analysis, cultures were pre-confluent (24 h in FBS) or post-confluent (72 h in FBS). At the time of harvest, approximately 30% of cells in pre-confluent control cultures were in G0/G1 (Figure 3) and less than 20% were in G2/M. Colchicine treatment of pre-confluent cultures increased ($P < .05$) the proportion of G2/M cells with primarily a concomitant decrease in S phase cells (Figure 3).

Approximately 80% of cells in post-confluent control cultures were in G0/G1 (Figure 3), compared with

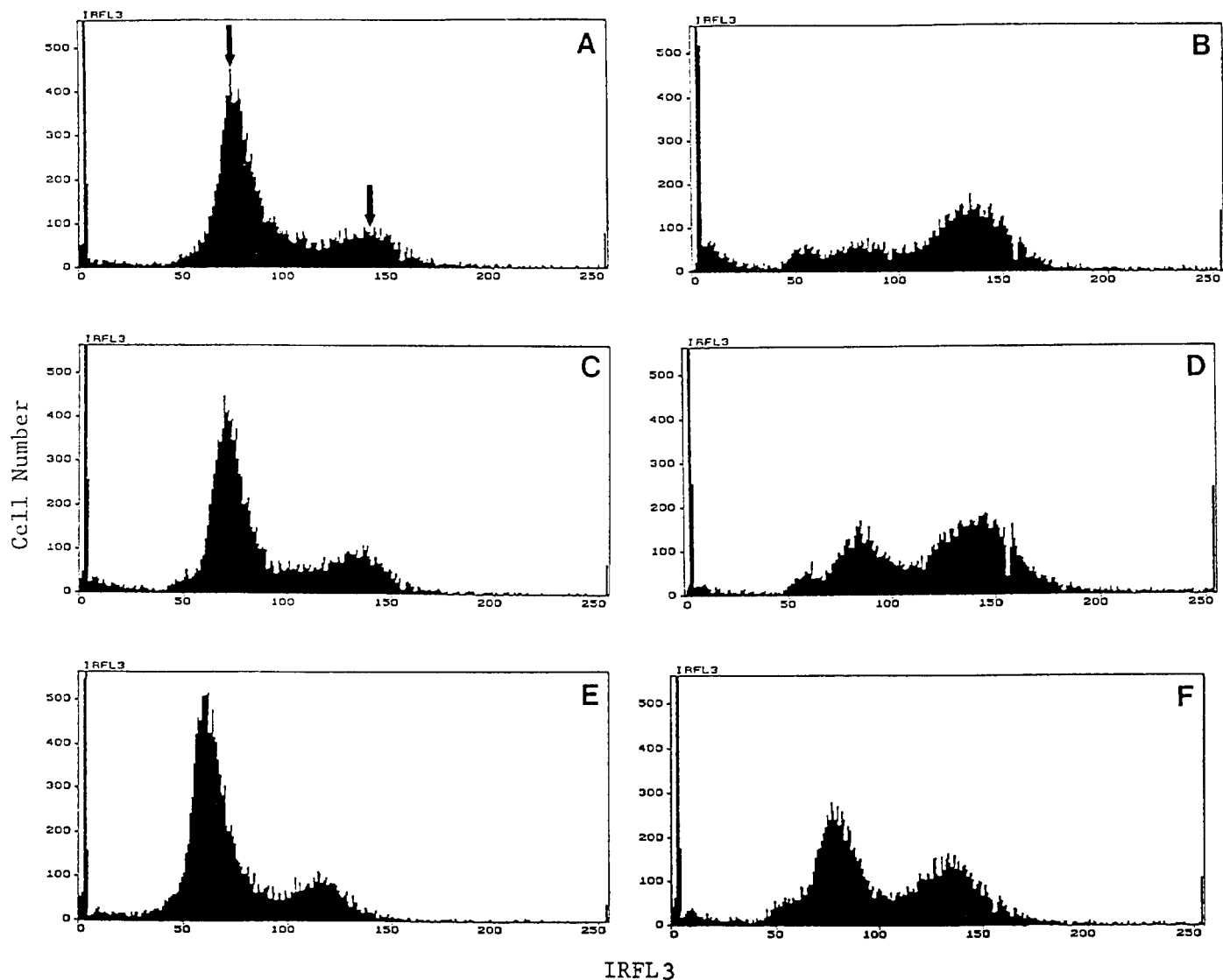


Figure 1. Flow cytometric analysis of DNA content of cells cultured in fetal bovine serum (FBS). Cells were grown for 3 d in medium supplemented with FBS. Histograms depict cell number vs propidium iodide staining (IRFL3) of .5 \times (A,B), 1 \times (C,D), and 2 \times (E,F) cultures. Cultures were pre-confluent (.5 \times) to post-confluent (2 \times) at the time of analysis. Profiles B, D, and F depict cultures treated with colchicine 24 h before harvest. Arrows in panel A mark post-mitotic or 2N (G0/G1) and G2/M (mitotic or 4N) peaks. For quantitative purposes (Figure 2), mean fluorescence values were 72 (range from 51 to 96) for G0/G1 cells, 138 (range from 120 to 179) for G2/M cells, and the median value of 107 (range from 96 to 118) for S (in DNA synthesis but < 4N) cells.

only 30% in pre-confluent control cultures. Colchicine treatment did not significantly increase the proportion of G2/M cells but caused a moderate but significant decrease in G0/G1 cells (Figure 3).

AD-1 Antigen Expression and DNA Analysis of Stromal Vascular Cells

To determine whether preadipocytes could be identified concomitant with PI staining, S-V cells were cultured for 72 h in medium supplemented with 10% FBS and an additional 24 h in medium supplemented with 2 to 10% PS. Increasing concentrations of PS were used in this series of experiments because prior

to differentiation or lipid deposition, the proportion of AD-1 positive cells in S-V cultures increases over time in the presence of PS (Wright and Hausman, 1990a; Wright, 1992). All cultures were confluent at the time of analysis. In controls, the relative contribution of PI staining toward FITC immunofluorescence, and vice versa, was examined. There was no background FITC staining due to PI or background PI staining due to FITC (unpublished results).

Representative two-parameter, three-dimensional histograms of cells grown in 2% (A,B), 5% (C), and 10% (D) PS are shown in Figure 4. Control profile (Figure 4A) demonstrates positive PI staining

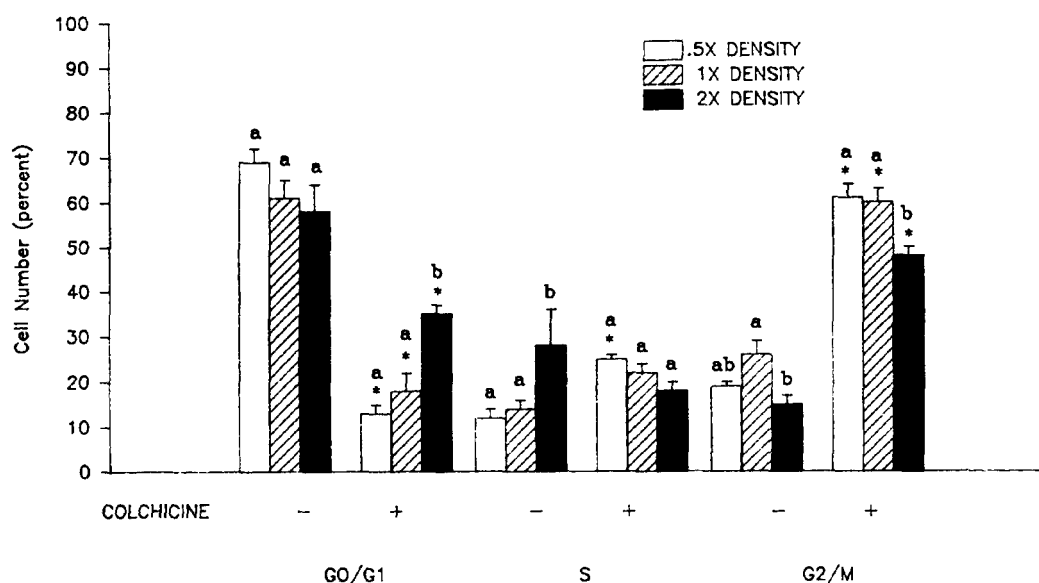


Figure 2. Flow cytometric analysis of DNA content of cells cultured in fetal bovine serum (FBS). Culture conditions are the same as in Figure 1. The cell number (percentage of total cells) represents mean \pm SE of four separate experiments. Statistical comparisons were within each phase and not among cell cycle phases and included comparisons of control vs colchicine treatment within a given density (*) and comparison among densities within treatment (superscripts). Colchicine treatment means with an asterisk are different from density and cell cycle-matched control ($P < .05$). Means across densities with different superscripts are different ($P < .05$) within treatment and cell cycle. Cell cycle phases indicated are G0/G1 (post-mitotic or 2N cells), S (cells synthesizing DNA but $< 4N$), and G2/M (mitotic cells that are 4N).

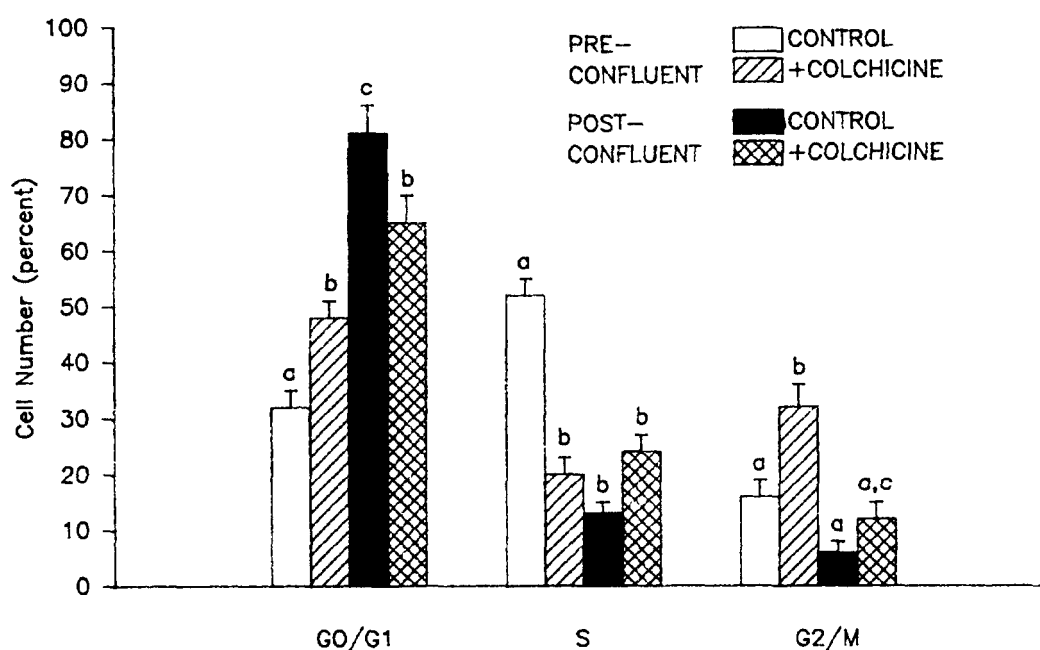


Figure 3. Flow cytometric analysis of DNA content of cells in pre-confluent and post-confluent cultures in differentiation medium. Cells were seeded at 1X density and grown for either 24 h (pre-confluent) or 72 h (post-confluent) in medium supplemented with 10% fetal bovine serum (FBS) and an additional 24 h in medium supplemented with 2% pig serum with 10 nM dexamethasone (PS/DEX). The cell number (percent) represents mean \pm SE of four separate experiments. Statistical comparisons were within and not among cell cycle phases. Means with different superscripts are different ($P < .05$) within cell cycle phase. Cell cycle phases indicated are G0/G1 (post-mitotic or 2N cells), S (cells synthesizing DNA but $< 4N$), and G2/M (mitotic cells that are 4N).

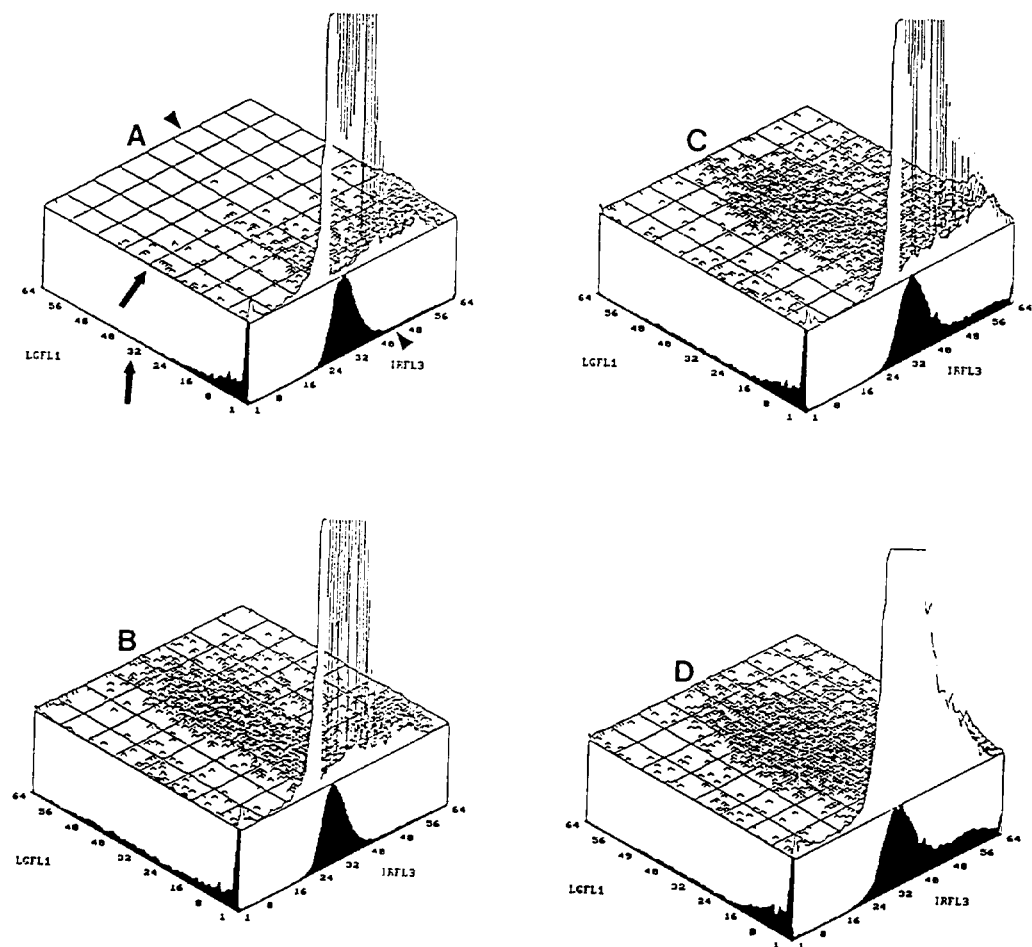


Figure 4. Two-parameter flow cytometric analysis of DNA content and AD-1 (anti-adipocyte/preadipocyte monoclonal antibody) antigen expression by cultured stromal-vascular cells. Cells were cultured for 72 h in medium supplemented with 10% fetal bovine serum (FBS) and an additional 24 h in medium supplemented with 2% (A,B), 5% (C), and 10% (D) pig serum (PS). Representative profiles depict AD-1 immunofluorescence (LGFL1) vs propidium iodide (PI) fluorescence (IRFL3) plotted against cell number on the third axis. Control profile (A) demonstrates PI staining vs background staining using a negative control monoclonal antibody. Arrows mark position of background fluorescence using negative monoclonal antibody and arrowheads mark upper limit of S (in DNA synthesis but < 4N) phase cells.

Table 1. Two-parameter flow cytometric analysis of DNA content and AD-1 antigen expression^a

Treatment	Proportional cell number (% ± SE)			
	G0/G1-S ^{bc}	G2/M ^d	AD-1	G2/M, AD-1 ^e
2% Pig serum	80 ± 4	20 ± 3	8 ± 2	2 ± 1
5% Pig serum	71 ± 5	29 ± 4	8 ± 2	3 ± 2
10% Pig serum	62 ± 4	38 ± 4	11 ± 2	6 ± 2

^aData represent mean ± SE of three separate experiments.
^bG0/G1 = post-mitotic, 2N cells.
^cS = cells in DNA synthesis but < 4N.
^dG2/M = cells (4N) immediately prior to mitosis.
^eAD-1 = monoclonal anti-adipocyte/preadipocyte antibody.
Copyright © 1994 American Society of Animal Science. All rights reserved. For personal use only. No other uses without permission.

(IRFL3) vs background FITC staining (LGFL1) using a negative control MAb. At the three serum concentrations used (Figure 4B-D), from 8 to 11% of the S-V cells were AD-1 positive (LGFL1 > 32, arrows) or preadipocytes (Table 1). In 2% PS cultures (Figure 4, Table 1), approximately 80% of the cells were in G0/G1 and S (IRFL3 < 49). A serum-dependent decrease in G0/G1 cells and increase in G2/M cells was observed upon increasing the concentration of PS from 2 to 10% (Table 1). In all instances, AD-1 immunoreactive cells were predominantly in, but not restricted to, the G0/G1-S cell subpopulations (Figure 4). The number of G2/M-AD-1 positive cells (LGFL1 > 32, IRFL3 > 49, Figure 4) were from 2 to 6% of the total cells (Table 1) or approximately 25 to 50% of the AD-1 positive cells (G2/M, AD-1/AD-1, Table 1), depending on serum concentration.

Discussion

The cell cycle of BALB/c-3T3 T cells has been studied in some detail by autoradiographic analysis of DNA synthesis following culture in [³H]thymidine (Hoerl and Scott, 1989). Moreover, proliferation of primary S-V cells in vitro has been quantified in several ways, including measurement of radioactive thymidine incorporation into DNA (Ramsay et al., 1987, 1989). By labeling cells at different times followed by separating lipid-filled adipocytes using Percoll, it has also been possible to measure replication of preadipocytes per se (Ramsay et al., 1989). These techniques, although indirect, do offer some information concerning proliferation. However, with primary (non-cell line) preadipocytes, it is impossible to determine whether preadipocytes are replicating preferentially because of the difficulty of specifically labeling and identifying replicating precursors.

We have divided cultured porcine S-V cells into three subpopulations based on DNA content or G0/G1, S, and G2/M cells. The G0/G1 and G2/M subpopulations are readily identified based on DNA content. Conversely, S phase cells, for the purpose of this study, are apparent only after extrapolation of computer-forced baseline of G0/G1 and G2/M. The cell cycle is much more complex on closer examination. However, because of the absence of current data, the importance of investigating even the most basic aspects of preadipocyte replication is apparent.

The algorithm presented in this study demonstrated that proliferation of primary S-V cells can be measured by PI staining of DNA followed by flow cytometry. Furthermore, the small proportion of replicating preadipocytes (AD-1 positive) was also measured by simultaneously labeling cells with the anti-adipocyte/preadipocyte MAb. Treatment with colchicine to inhibit mitosis verified the identity of G2/M cells. In no instance did all cells accumulate at G2/M

after colchicine treatment. Treatment for an additional 24 h caused only a moderate increase in G2/M cells over the number observed at 24 h and also resulted in detachment of some cells from the substratum (unpublished data). Therefore, in these experiments either not all S-V cells were mitogenically active, or possibly some were dividing at a sufficiently slow rate so as not to reach G2/M within the 24-h colchicine treatment period. Treatment of cultures with increasing concentrations of PS increased replication (G2/M cells) without a concomitant change in the proportion of AD-1 positive cells, suggesting that the mitogenic effect of PS is not specific for either preadipocytes or non-preadipocytes in cultures.

Cells grown for 3 d in FBS responded strongly to colchicine treatment at each of the three seeding densities (Figure 2). The majority of cells were actively replicating even at higher, post-confluent densities, most likely because of the mitogenic nature of FBS on S-V cells. In all FBS cultures, the increase in G2/M cells in response to colchicine treatment occurred concomitant with a decrease in G0/G1 cells, suggesting that under these conditions the vast majority of G0/G1 cells were probably G1 cells (as opposed to G0), and therefore replicating. Additionally, the decrease in G0/G1 cells in response to colchicine treatment was greater in FBS cultures than in DEX cultures. In pre-confluent PS/DEX cultures treated with colchicine (Figure 3), an increase in G2/M cells was due primarily to a decrease in S phase cells, as opposed to a decrease in G0/G1 cells, as seen in FBS cultures. Therefore, prior DEX exposure may have resulted in the majority of cells withdrawing from the cell cycle (G0 or quiescent), but S cells were not prevented from accumulating in G2/M. The anti-mitogenic effect of DEX, therefore, seems generalized and without any apparent consequence to stromal vascular cells (non-preadipocytes) in cultures. After cells begin to replicate DNA, they complete that round of synthesis and go on to mitosis. It will be of interest to examine specific effects of growth factors on primary S-V cells to determine their ability to induce replication in G0/G1 cells and G0 preadipocytes. Recently, reduction in preadipocyte number has been observed in cultures prepared from hypophysectomized fetuses (Wright and Hausman, 1993). Propidium iodide staining and flow cytometry would be useful in understanding how hypophysectomy affects preadipocyte number.

Implications

With specific cell surface markers such as anti-adipocyte/preadipocyte monoclonal antibodies, it is now possible to examine the specific, primary preadipocyte cell subset. For example, cells positive for

the AD-1 anti-adipocyte/preadipocyte monoclonal antibody can be quantified directly after growth under numerous conditions (Wright, 1992). Also, increases in the number of immunoreactive cells can be correlated with replication (present study). Application of this technique to in vivo models of adipose tissue hyperplasia is important because regulation of preadipocyte proliferation is a critical factor in determining adipose tissue mass.

Literature Cited

- Bjorntorp, P., M. Karlsson, L. Gustafsson, U. Smith, L. Sjostrom, M. Cigoloni, G. Storck, and P. Pettersson. 1979. Quantitation of different cells in the epididymal fat pad of the rat. *J. Lipid Res.* 20:97.
- Gaskins, H. R., J.-W. Kim, and G. J. Hausman. 1990. A decrease in local hormone biosynthesis and c-Fos gene expression accompany differentiation of porcine preadipocytes. *In Vitro Cell and Dev. Biol.* 26:1049.
- Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: Controls that ensure the order of cell cycle events. *Science* (Washington, DC) 246:629.
- Hentges, E. J., and G. J. Hausman. 1989. Primary cultures of stromal-vascular cells from pig adipose tissue: The influence of glucocorticoids and insulin as inducers of adipocyte differentiation. *Domest. Anim. Endocrinol.* 6:275.
- Hoerl, B. J., and R. E. Scott. 1989. Nonterminally differentiated cells express decreased growth factor responsiveness. *J. Cell Physiol.* 139:68.
- Ramsay, T. G., G. J. Hausman, and R. J. Martin. 1987. Preadipocyte proliferation and differentiation in response to hormone supplementation of decapitated fetal pig sera. *J. Anim. Sci.* 64:735.
- Ramsay, T. G., C. K. Wolverson, G. J. Hausman, R. R. Kraeling, and R. J. Martin. 1989. Alterations in adipogenic and mitogenic activity of porcine serum in response to hypophysectomy. *Endocrinology* 124:2268.
- SAS. 1985. SAS User's Guide: Statistics (Version 5 Ed.). SAS Inst. Inc., Cary, NC.
- Wright, J. T. 1992. Flow cytometric analysis of porcine preadipocytes. *J. Cell Biochem.* 48:385.
- Wright, J. T., and G. J. Hausman. 1990a. Monoclonal antibodies against cell surface antigens expressed during porcine adipocyte differentiation. *Int. J. Obes.* 14:395.
- Wright, J. T., and G. J. Hausman. 1990b. Adipose tissue development in the fetal pig examined using monoclonal antibodies. *J. Anim. Sci.* 68:1170.
- Wright, J. T., and G. J. Hausman. 1993. In vitro differentiation of preadipocytes from hypophysectomized pig fetuses. *J. Anim. Sci.* 71:1447.

Citations

This article has been cited by 1 HighWire-hosted articles:
<http://jas.fass.org#otherarticles>